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13. ABSTRACT *(Maximum 200 words)*

Macrophages secrete interferons (IFNs), as well as other inflammatory cytokines, following stimulation with lipopolysaccharide (LPS), the outer membrane component of Gram negative bacteria that has been implicated as the initiator of the sepsis-associated Systemic Inflammatory Response Syndrome (SIRS). The interferon regulatory factors (IRFs) comprise a family of DNA binding proteins that positively and negtively regulate transcription of IFN and certain IFN-inducible genes. Basal and LPS-inducible levels of mRNA expressed by three IRF family member genes, i.e., IRF-1, IRF-2, and ICSBP, as well as a panel of other well characterized, SIRS-associated, inflammatory genes, were analyzed in macrophages derived from fully LPS-responsive mouse strains (*Lps*^{n}), genetically LPS-hyporesponsive (*Lps*^{d}) mice, IRF-1 and IRF-2 "knockout" mice, as well as from *Lps*^{n} macrophages rendered "endotoxin tolerant" *in vitro*. Our results suggest that the IRF nuclear binding proteins, as well as serine/threonine phosphatases, play important roles in LPS-induced gene expression and may provide novel targets for therapeutic intervention, not only in Gram negative sepsis, but also in other syndromes characterized by inflammatory mediator excess.

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FINAL REPORT

GRANT #: AASERT N00014-93-MP2F012 (ONR MDA 905-93-Z-0010)

INSTITUTION: Department of Microbiology and Immunology
Uniformed Services University of the Health Sciences

GRANT TITLE: Regulation of Interferon Regulatory Factors in LPS-Stimulated Macrophages

AWARD PERIOD: 5/28/93 - 12/31/95

PI: Dr. Stefanie N. Vogel **AASERT Fellow:** Sheila A. Barber

OBJECTIVE: The basic objective of the work carried out by the AASERT student was to characterize the participation of Interferon Regulatory Factors (IRFs) in the intracellular signaling pathways induced in murine macrophages by lipopolysaccharide (LPS). In addition, the molecular regulation of the expression of specific IRFs in response to LPS was examined.

APPROACH: Northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) technologies were used to measure quantitative changes in mRNA expression for a panel of LPS-inducible genes, including the IRFs, in LPS-responsive and LPS-hyporesponsive murine macrophages. In addition, Western blot analysis was carried out to examine LPS-induced phosphorylation on tyrosine residues.

ACCOMPLISHMENTS:

Macrophages secrete IFN, as well as other cytokines, following LPS stimulation. The interferon regulatory factors (IRFs) comprise a family of DNA binding proteins that have been implicated in the transcriptional regulation of IFN and certain IFN-inducible genes. We therefore characterized basal and LPS-inducible levels of IRF-1, IRF-2, and ICSBP mRNA in LPS-responsive macrophages and compared the expression of these genes in macrophages that typify two murine models of LPS-hyporesponsiveness. In the first model, the LPS-hyporesponsive phenotype of the C3H/HeJ mouse is genetically determined and maps to the *Lps* locus on mouse Chromosome 4. In the second model, normally LPS-responsive macrophages acquire a transient LPS-hyporesponsive phenotype following a prior exposure to LPS, a phenomenon referred to as "endotoxin tolerance." Using reverse transcriptase polymerase chain reaction, RT-PCR, we detected significantly higher basal levels of IFN-β mRNA in LPS-responsive (Lps^n) than LPS-hyporesponsive (Lps^d)macrophages, as well as basal levels of IRF-1 mRNA in Lps^n macrophages that were ~15-times higher than those found in Lps^d macrophages. Conversely, Lps^d macrophages expressed basal levels of IRF-2 mRNA that were ~18-times higher than those expressed in Lps^n macrophages. LPS stimulation resulted in a dose- and time-dependent accumulation of IRF-1, IRF-2, and ICSBP mRNA only in Lps^n macrophages. Cycloheximide inhibited the accumulation of LPS-stimulated IRF-2 and ICSBP mRNA, but not IRF-1 mRNA, thus designating IRF-1 an immediate-

early, LPS-inducible gene. Finally, endotoxin-tolerized macrophages expressed elevated, but non-maximal mRNA levels for all three transcription factors that are not re-induced upon secondary challenge with LPS. Thus, the IRFs represent yet an additional molecular pathway in the complex response to LPS. Finally, C3H/HeJ (*Lps*^{d}) macrophages were confirmed to express lower levels of not only IFN-β, but also, IFN-α and IFN-γ mRNA. Subsequent experiments demonstrated that IFN-α could substitute for LPS in C3H/HeJ mice as a "priming" signal for the induction of IFN by poly I:C.

Preliminary experiments were also carried out on macrophages derived from mice with targeted disruptions in the IRF-1 or IRF-2 gene, i.e., IRF-1 and IRF-2 "knockout mice" (IRF-1$^{-/-}$ and IRF-2$^{-/-}$, respectively). Our ability to procure these animals came about as Dr. Barber's project was nearing completion and the number of mice made available to us from Dr. Tak Mak (Amgen, Ontario) was very limited, thus precluding a complete analysis of the macrophage response to LPS. However, some important general trends were observed which are currently being confirmed and extended by additional experiments in our laboratory. The panel of LPS-inducible genes examined included IFN-β, IRF-1, IRF-2, ICSBP, as well as TNFα, IL-1β, IP-10, and TNFR-2. The findings can be summarized as follows: (1) There was no difference in basal level expression of this panel of genes in macrophages derived from IRF-1$^{-/-}$ and control C57BL/6 mice in medium-treated controls or 1 - 3 hr LPS-treated time points, with the exception that IRF-1$^{-/-}$ macrophages expressed lower basal levels of IFN-β mRNA in 5 of 6 separate comparisons (IRF-1 levels were not measured in the IRF-1$^{-/-}$ macrophages). (2) LPS-inducible TNFα gene expression was the least affected by targeted disruption of the IRF-1 gene; and (3) At later time points (i.e., after 4-6 hr), the expression of IL-1β, IP-10, TNFR-2, and ICSBP genes was lower in IRF-1$^{-/-}$ macrophages than in C57BL/6 controls; and, (4) IRF-2 and IFN-β gene expression was reduced in IRF-1$^{-/-}$ macrophages after 7-9 h of LPS stimulation. The IRF-2$^{-/-}$ macrophages exhibited the following general responses: (1) There was no difference in basal level expression of any gene examined, with the possible exception of IFN-β, where IRF-2$^{-/-}$ macrophages exhibited slightly higher basal levels of IFN-β (IRF-2 gene expression was not measured in the case of the IRF-2$^{-/-}$ macrophages); (2) IRF-1 and IFN-β gene expression in LPS-stimulated macrophages was very similar to that observed in the control macrophages; (3) TNF-α, IL-1β, IP-10, and ICSBP gene expression was lower after 4 hr of LPS stimulation in IRF-2$^{-/-}$ macrophages, compared to the C57BL/6 controls, while the expression of TNFR-2 was lower in the IRF-2$^{-/-}$ macrophages essentially throughout the time course.

Macrophages respond to LPS with rapid protein phosphorylation and dephosphorylation on serine, threonine, and tyrosine residues, and phosphorylation of nuclear binding proteins, including the IRF family members, has been shown to be critical for transactivating/transrepressing activities of these molecules. If these events are critical for the cellular response to LPS, the kinases and/or phosphatases involved may be vulnerable targets for pharmacologic intervention. Recent studies demonstrated that tyrosine kinase inhibitors block LPS-induced tyrosine phosphorylation of MAP kinases as well as TNF-α and IL-1β production. To investigate a role for serine/threonine phosphatases, we evaluated the effect of calyculin A, a potent inhibitor of protein phosphatase (PP) PP1 and PP2A, two of the

most abundant serine/threonine phosphatases in the cell, on LPS stimulation of murine macrophages. Pretreatment of macrophages with calyculin A inhibits LPS-induced expression of 6 immediate-early genes: TNF-α, IL-1β, IFN-β, IP-10, IRF-1, and TNFR-2. Calyculin A added 1.5 h after LPS treatment greatly reduced accumulation of IP-10, IRF-1, and TNFR-2 mRNA, but not TNF-α , IL-1β, or IFN-β mRNA. Calyculin A, in the absence or presence of LPS, results in sustained tyrosine phosphorylation of the MAP kinases. These findings suggest that an "early" serine/threonine phosphatase activity is essential for LPS stimulation of macrophages and that the activation of MAP kinases is not sufficient for the induction of these immediate-early genes. The requirement for a "late" phosphatase activity for expression of a subset of LPS-inducible genes dissociates at least two regulatory pathways in LPS signal transduction.

In contrast to the broad serine/threonine phosphatase activity exhibited by calyculin A, okadaic acid is more selective for protein phosphatase 2A. Its effects on LPS-inducible genes revealed a distinct pattern of gene expression, suggesting that more selective inhibition of a subclass of serine/threonine phosphatases results in a distinct pattern of dysregulation of gene expression in the absence or presence of LPS. Specifically, okadaic acid was found to induce IL-1β, TNFα, IL-6, IFN-β, and IP-10, but not IL-10 or IL-12 (p40) mRNA. Okadaic acid was found to regulate the expression of LPS-inducible IL-10 and IL-12 (p40) differentially. These findings suggest that okadaic acid-sensitive phosphatases are key regulators of cytokine production in unstimulated and activated macrophages. Finally, okadaic acid inhibits inducible nitric oxide synthase (iNOS) mRNA and nitric oxide production by macrophages activated by LPS and IFN-γ. Coordinate inhibition by okadaic acid of IRF-1 mRNA, shown in previous studies to be essential for the expression of iNOS mRNA, suggests that the inhibition of iNOS mRNA by the serine/phosphatase inhibitor may due to reduced transcriptional expression of the nuclear transactivating protein, IRF-1.

Lipid second messengers are gaining recognition as important mediators of extracellular signals. One such lipid, ceramide, generated from membrane sphingomyelin following stimulation with TNF-α, IL-1β, or IFN- γ, activates ceramide-activated kinase (CAK). A recent study demonstrated that LPS activates CAK without generating ceramide, suggesting that the LPS stimulation of cells mimics the second messenger function of ceramide. To compare ceramide to LPS signaling, we assessed the ability of LPS-responsive (*Lps*^{n}) and LPS-hyporesponsive (*Lps*^{d}) macrophages to respond directly to ceramide for enhanced expression of LPS-inducible genes. In contrast to macrophages from C3H/OuJ (*Lps*^{n}) mice, C3H/HeJ (*Lps*^{d}) macrophages failed to respond to cell-permeable analogs of ceramide (C_2,C_6,C_{16}) or sphingomyelinase (SMase). These results suggest that a common critical molecule, encoded by the *Lps* gene, regulates both ceramide and LPS signaling pathways.

To characterize further the LPS-mimetic effect of activating the ceramide pathway, macrophages were stimulated with LPS, ceramide analogs, or SMase and cytokine secretion, gene expression, and the ability to induce *in vitro* endotoxin tolerance were measured. SMase and LPS induced comparable levels of secreted TNF-α; however, unlike LPS, SMase failed to stimulate detectable IFN bioactivity.

Ceramide analogs induced the expression of many LPS-inducible genes, including TNFα, IRF-1, IRF-2, IL-1β, and TNFR-2, to levels equivalent or greater to those induced by LPS; however, expression of IP-10 and another IRF family member, ICSBP, was significantly lower than that induced by LPS. As was observed for LPS, pretreatment of macrophages with calyculin A inhibited the ability of SMase to induce TNFα secretion. Finally, macrophages pre-exposed *in vitro* to LPS to induce a well characterized state of "endotoxin tolerance" secreted little or no TNFα upon secondary "challenge" with either LPS or SMase, whereas macrophages pre-exposed to SMase were not tolerized to either LPS or SMase. Collectively, these results suggest that ceramide activates a subset of LPS-induced signaling pathways in macrophages.

CONCLUSIONS: The IRF family of nuclear binding proteins are induced by LPS and participate in the LPS-induced signaling pathway that leads to expression of a significant number of inflammatory genes. Not only are these genes expressed in response to an LPS exposure, but also in animals rendered septic. The products of these genes are felt to mediate the systemic inflammatory response syndrome that results ultimately in multisystem organ failure and death.

SIGNIFICANCE: The identification of IRF family members as participatory nuclear binding proteins in the LPS-induced response provides the basis for continued studies that target these factors as a potential intervention strategy in sepsis or other shock-like syndromes, including hemorrhagic shock.

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